Supplementary Material

Cytokine measurements in human PBMCs

a. Cells and cell cultures

HT-29 cells originate from a human colon adenocarcinoma from a 44-year-old, Caucasian female (ATCC, Manassas, USA). PBMCs were isolated from leukocyte filters, acquired from anonymous adult blood bank donors from the Institute for Transfusion Medicine of the Charité University, Berlin, Germany. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (PAA Laboratories GmbH, Pasching, Austria), supplemented with 4.5 g/l D-Glucose, 2 mM L-glutamine, 10% fetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany), 100 IU/ml penicillin, and 100 µM streptomycin (Life Technologies, Carlsbad, USA), designated as growth medium (GM). Cell cultures were grown at 37°C in a humidified atmosphere of 5% CO_2. HT-29 cells were grown in cell culture flasks; medium changes were performed 3 times a week.

b. Fatty acid preparation

EPA, DHA, and AA (Nu-Chek-Prep Inc., Elysian, USA) were dissolved in 100% ethanol to form stock solutions (100 mM and 1 mM) and stored at -20°C until further usage. PUFAs used for incubation with PBMCs were evaporated and resuspended in growth medium to remove potential confounding effects of residual ethanol. In this context, nitrogen gas was used to evaporate ethanol to prevent oxidation of PUFAs.

c. Acquisition of HT-29 cell supernatants (CM)

CM was collected during medium changes and centrifuged for 10 min at 3000 RPM, RT, to remove excess cell debris. The supernatants of medium changes were pooled, vortex-mixed, and stored at -20°C.

d. Isolation of peripheral blood mononuclear cells

Leukocyte depletion filters (LST2B, Maco Pharma International, Langen, Germany) were obtained from blood collection systems from the Institute for Transfusion Medicine of the Charité University, Berlin, Germany. Isolation of PBMCs was commenced within 2.5 hours after blood donation. For this, filters were flushed with 40 ml of PBS (Merck KGaA, Darmstadt, Germany) by attaching a sterile 20 ml syringe to one end of the blood bag system. The other end was placed over a cell culture flask and secured with a clip. Residual buffer was obtained by flushing the system with air, thus enhancing the PBMC yield. An average of approximately 80 ml of PBS-filter-suspension was obtained per filter. Subsequently, three 50 ml Falcon tubes were filled with 20 ml of Ficoll-Paque™ (GE Healthcare, Little Chalfont, UK) and 25 ml of the cell suspension were carefully overlaid onto the Ficoll-Paque™. The Falcon tubes were centrifuged for 20 min at 2200 RPM, RT, without break.

After centrifugation, PBMC buffy coats were collected from the interphase and transferred to a 50 ml Falcon tube. PBMCs were washed once with 30 ml of PBS. After allowing time to soak, cells were centrifuged at 1500 RPM for 5 min, 4°C. The supernatant was discarded and 3 ml of RBC Lysis Buffer was added to cells to remove residual erythrocytes. After 08:30 min, 20 ml of GM was added to stop the reaction. The obtained cell suspension was then filtered through a cell strainer (Thermo Fischer Scientific Inc., Waltham, USA). The cell strainer was rinsed with 15 ml of PBS and the resulting cell suspension was centrifuged at 1500 RPM for 5 min, 4°C. PBMCs were washed twice with 25 ml of PBS. After each wash step, PBMCs were centrifuged at 1500 RPM for 5 min, 4°C. Subsequently, PBMCs were counted and assessed for viability using trypan blue exclusion and diluted in GM to a concentration of $5*10^6$ cells/ml.

e. Fatty acid incubation and subsequent stimulation of peripheral blood mononuclear cells

2.5 $*10^6$ PBMCs were incubated with 500 µl of untreated GM or GM supplemented with 50 µM of DHA, EPA, or AA. PBMCs from day 1 were either supplemented with 500 µl of untreated GM

(termed PUFA-only (i.e. EPA-, DHA-, and AA-only)) or stimulated with either LPS (Sigma-Aldrich, St. Louis, USA) dissolved in 500 µl of GM (with a final concentration of 1 ng/ml/well) or 500 µl of CM. On day 3 cells and supernatants were harvested and transferred into 2.0 ml Eppendorf vials. The vials were centrifuged for 5 min at 3000 RPM, 4°C to remove cells and debris. Supernatants (1300 µl) were collected in new vials and stored at -20°C until further usage.
For controls, supernatants of untreated PBMCs and PBMCs stimulated with LPS or CM on day 2 (no prior PUFA incubation; termed LPS and CM controls) were used. All groups, except PUFA-only samples, were set up in triplicates. PUFA-only experiments were set up in single cell cultures.

f. Cytokine analysis / ELISA

TNF-α, IL-6, and IL-10 concentrations of supernatants were measured by using specific ELISA kits ("Ready-Set-Go!", eBioscience Inc., San Diego, USA). Samples were measured in duplicates and as outlined in the guideline provided by the manufacturer. Absorption of wells was measured by an EL800 universal Microplate Reader (Bio-Tek instruments Inc., Winooski, USA), using a wavelength of 450 nm. Supernatants and standards were diluted in GM. GM was also used for blank measurements. The supernatants were diluted so that the measured cytokine concentrations were to lie in-between standard curve range of the kits.

g. Controls

Cytokine levels found in supernatants of PBMCs incubated with EPA, DHA, or AA without subsequent stimulation (i.e. PUFA-only) did not vary significantly when compared to untreated PBMCs or with each other (data not shown). Mean cytokine levels for TNF-α and IL-10 of all PUFA-only samples were significantly lower than supernatants of cells stimulated with LPS or CM ($p < 0.01$, data not shown). Incubation of PBMCs with AA (i.e. AA-only) showed a tendency to increase IL-6 levels. These were not statistically significant when compared to untreated PBMCs or other PUFA-only samples (data not shown). Our results showed major differences in the absolute cytokine levels of different donors after stimulation with LPS or CM. We therefore standardized the data by expressing cytokine levels as fold-increase of LPS- or CM-controls.

h. Standardization & statistics

Statistical analysis was performed with GraphPad Prism 6.0f Software (GraphPad Software Inc., La Jolla, USA). Probability values of $p < 0.05$ were considered as significant. The analysis was verified by the Department of Statistics at Ludwig Maximilians University, Munich, Germany.
Results are standardized as the mean ± SEM of ratios calculated for each individual patient. These ratios show the relative change of cytokines compared to LPS or CM controls. The mean ratios from different experimental groups were compared to controls using Students t-test. Differences between experimental groups (EPA, DHA, and AA) were analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. The results represent data obtained from 5 PBMC donors.